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Mental Status and Fragile X Expression in Relation to FMR-1 Gene Mutation

Abstract

The fragile X mental retardation syndrome is caused by unstable expansion of a CGG repeat in the FMR-1 gene. Clinical expression is associated with a large expansion of the CGG repeat. The mutation in the FMR-1 gene and the cytogenetic expression of the fragile site at Xq27.3 have been studied in 52 fragile X male patients. The percentage of the cytogenetic expression of the fragile site at Xq27.3 positively correlates with the mean size of the full mutation in the FMR-1 gene ($p < 0.0001$) irrespective of the presence of additional premutation alleles. We noted a less frequent occurrence of additional premutation alleles in adult patients compared with juveniles, suggesting a continued mitotic instability in life. Additionally, the level of mental retardation has been ascertained in 35 patients using the Stanford-Binet or Terman-Merrill test of general intelligence. The presence of a full mutation in the FMR-1 gene seemed decisive for the occurrence of mental impairment in the patient. No correlation is observed between the degree of mental retardation and the size of the full mutation. The degree of mental retardation seemed not to be influenced by the presence of premutation alleles in part of the cells in addition to a full mutation. One patient is described with the 'Prader-Willi-like' subphenotype of the fragile X syndrome, showing a deletion in the FMR-1 gene in a part of his cells in addition to a full mutation.

Key Words

Fragile X syndrome
FMR-1
Methylation
Mental status
Cytogenetic expression
Deletion

Introduction

The fragile X syndrome is the most common cause of familial mental retardation. Its prevalence in Western societies is estimated to be 1/1,250 for affected males and 1/2,000 for females [1, 2]. Most affected males have as main clinical features: mental retardation, macroorchidism and a long narrow face with everted ears which is designated as the Martin-Bell phenotype [3–5]. The clinical diagnosis in male patients can be confirmed by cytogenetic testing, showing the fragile site at Xq27.3 in 2–60% of the cells [6, 7].

Recently, a gene (FMR-1) related to the mental retardation was isolated [8]. It was shown that the fragile X syndrome is caused by elongation of a small DNA fragment, containing a repeat of the trinucleotide CGG located in the 5' exon of the FMR-1 gene [8–12]. Most likely, the increase in size is due to amplification of this repeat sequence. Normal X chromosomes have between 6 and 46 copies of CGG, which are stable during meiosis and a nearby CpG island is unmethylated on active X chromosomes [12]. Premutation alleles are characterized by an increase in the number of triplets to 52–200. Premutations do not cause mental retardation, the carrier males or females do not show cytogenetic expression of the fragile site and the CpG island remains unmethylated. This pre-mutation sequence is unstable during meiosis. In the full mutation, the repeat sequences exceed the size of 200 triplets and often show somatic heterogeneity; the fragile site becomes apparent in cytogenetic testing in males as do the clinical features of the fragile X syndrome. The CpG island has become methylated. About 15% of patients have a full mutation in the majority of their cells but carry a pre-mutation in a minority of the cells [9].

Several studies have shown that the majority (2/3) of the male fragile X patients are

moderately or severely retarded [13]. However, in fragile X patients, profound or borderline mental retardation have also been reported. Cross-sectional and longitudinal data indicate that IQ levels diminish with age in most patients. The majority of the prepubertal boys function at a moderate level (IQ \pm 35–55) and most of the adults are severely retarded (IQ \pm 20–35) [13]. This decline in IQ does not indicate a loss of mental capacities, but merely represents a slowing of cognitive development, resulting in a stabilisation of intellectual abilities after puberty [14–16].

We investigated whether there is a correlation between the percentage of cytogenetic expression of the fragile site at Xq27.3 and the insert size in the FMR-1 gene. Secondly, we questioned whether there is a relation between the mental capacities of the fragile X patient versus the type of mutation in the FMR-1 gene.

Materials and Methods

Fifty-two mentally retarded boys and adults in whom fragile X syndrome was confirmed by cytogenetic expression of the fragile site at Xq27.3 were included in the study.

DNA Analysis

The intragenic DNA probe pP2 was used for DNA analysis of the FMR-1 gene [17]. Genomic DNA was isolated from blood leukocytes [18]. DNA (8 μ g) was digested to completion with the restriction enzymes EcoRI or EcoRI and EagI according to the manufacturer's instructions, separated by gel electrophoresis and subjected to Southern blot analysis according to standard procedures [19]. The probe was labelled by the random oligonucleotide priming method [20]. After prehybridization and hybridization, the filters were washed to $0.1 \times$ SSC at 65 °C prior to autoradiography [19].

Cytogenetic Testing

Blood samples were cultivated under conditions designed to demonstrate the fragile site at Xq27.3 [7, 21]. At least 50 metaphase spreads were examined from each patient.

Determination of IQ Levels

IQ testing was performed on 35 patients, aged 2–26 years (mean 13.2 ± 6.7 years). Each individual had been tested at least once during the last year with the Stanford-Binet, or Terman-Merrill test of general intelligence [22] and a Dutch scale for adaptive functioning [23]. These two measures yielded the level of mental retardation according to the guidelines of the AAMD [24].

Almost all patients had been repeatedly tested with the Stanford-Binet test over an extended period which allowed identification of a significant decline in IQ scores. A significant decline is defined as a regression of at least 16 IQ points (= 1 SD).

Results and Discussion

FMR-1 Gene Mutation in Patients

DNA of fragile X patients was studied to determine the size of the insert in the FMR-1 gene. DNA analysis showed multiple fragments with different insertions larger than 500 bp that appear as a fuzzy band or smear on a Southern blot (fig. 1, lanes 2, 4, 5 and 7). The patients with the full mutation were all somatic mosaics for the insertion in the FMR-1 gene. Some of the fragile X patients had, in addition to the full mutation, a single abnormal band which corresponds to an insertion smaller than 500 bp and has the size of a premutation allele (fig. 1, lanes 3 and 6).

The patients can be classified into two groups according to their banding pattern: (1) full mutation (often appearing as a smear); (2) full mutation together with a premutation allele.

The second group consists of 28% of the patients. This number is higher than the 15% described by Rousseau et al. [25]. This may be explained by the relatively low mean age (19.1 ± 13.7) of our patients. In patients with an age < 20 years ($n = 35$) we observe 12 patients with a premutation (34%), while in the remaining patients ($n = 17$) we found only 2 patients with a premutation (11%) (Fischer's

exact test, $p = 0.06$). This suggests an age-dependent process whereby in adult males the premutation tends to expand completely to a full mutation due to continued mitotic instability in life.

There is a CpG island immediately proximal to the exon of the FMR-1 gene containing the insert and this is methylated in individuals with a full mutation who have the clinical symptoms of the fragile X syndrome [9]. The methylation level of this CpG island was studied by using the methylation-sensitive restriction enzyme EagI. The CpG island was completely methylated in the males with a full mutation. No fragments resulting from EagI activity were seen in DNA analysis of these males (fig. 2, lanes 2, 3, 4, 6 and 7). In all males with a premutation in addition to the full mutation, lack of methylation was seen in the premutation allele. DNA analysis of these males showed distinct fragments due to EagI activity (fig. 2, lanes 5 and 8).

FMR-1 Gene Mutation and Fragile X Expression

We studied in 36 male patients whether there is a relation between the percentage of cytogenetic expression of the fragile site at Xq27.3 and the mutation in the FMR-1 gene. Patients with a full mutation only ($n = 28$) had a cytogenetic expression between 2 and 60% (mean 23.1 ± 16.1). The patients with a full mutation and a premutation ($n = 9$) showed cytogenetic expression of the fragile site between 1 and 40% (mean 17.6 ± 11.5).

If the fragile X expression is influenced by the size of the inserts, one might expect a higher percentage of fragile X expression among the patients with full mutation in the FMR-1 gene in comparison with the patients who have an additional premutation. This may be conceivable because normal transmitting males have a premutation only in the FMR-1 gene in the absence of a cytogenetic

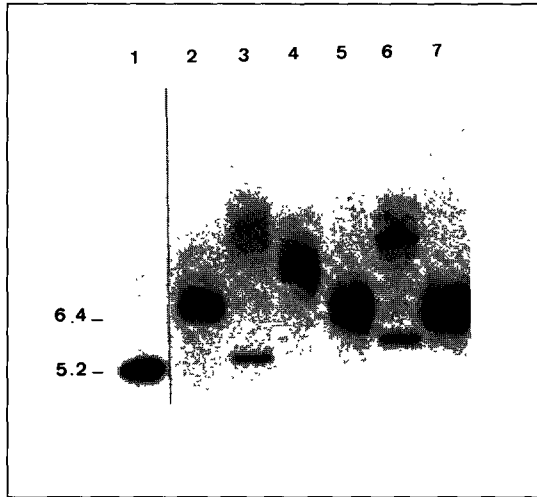


Fig. 1. Analysis with probe pP2 of EcoRI digested DNA of an unaffected male (lane 1) and 6 affected males (lanes 2-7). Patients 2, 4, 5 and 7 have a full mutation only, and patients 3 and 6 have a premutation in addition to a full mutation.

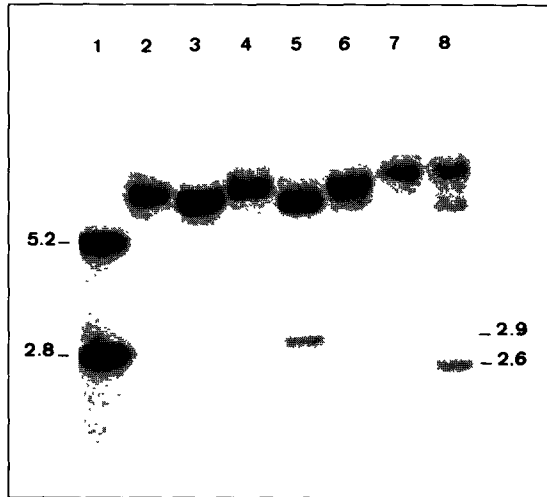


Fig. 2. Analysis with probe pP2 of EcoRI plus EagI digested DNA of a normal female and 7 affected males (lanes 2-8). The female (lane 1) shows a 2.8-kb band and a 5.2-kb band of the active and inactive X chromosome, respectively. Patient 5 has an unmethylated fragment with an insert of 100 bp and patient 8 has an unmethylated fragment with a deletion of 200 bp.

expression. Our results show that the mean percentage of fragile X expression is lower in the group with a premutation but the difference between both groups is not significant. The cells with a premutation account for a small minority of the total cells. The cells with large inserts appear to determine the fragile X expression, therefore a possible relation between the size of the full mutation and the percentage of fragile X expression was investigated. We examined the relation between the mean size of the inserts of the full mutation and the percentage of the fragile X expression in the same 36 patients. The mean insert size of the full mutation has been ascertained by averaging the smallest and largest detectable inserts. A positive correlation between the mean insert size and the cytogenetic expression was clearly demonstrated (fig. 3) ($r = 0.68$, $p < 0.0001$). In the CGG repeat a num-

ber of breakpoints are located that are detected in somatic cell hybrids with induced breakpoints in the fragile site [11, S.T. Warren, pers. commun.]. From fluorescence in situ hybridization experiments with probes crossing the fragile site it is suggested that fragile X expression is the result of a break in one of the chromatids [26]. Therefore, we conclude that the size of the insert is a causal factor in the generation of the fragile site and that the percentage of cytogenetic expression is determined by the size of the full mutation.

FMR-1 Gene Mutation and Mental Status

The mental status of the fragile X patients was studied in relation to the type of mutation in the FMR-1 gene in 35 boys and adult males with fragile X syndrome. This group is partly overlapping with the group of patients described in the earlier section. We compared

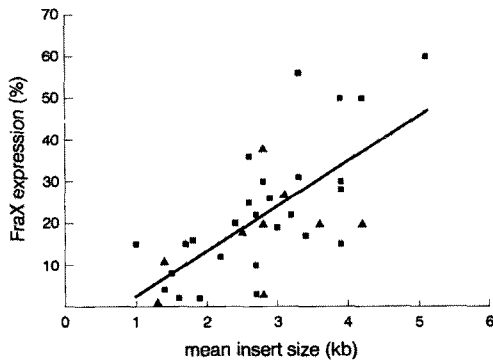


Fig. 3. Mean insert size of the full mutation in the FMR-1 gene related to the percentage of the fragile X expression in 36 patients. ■ = Patients with a full mutation only; ▲ = patients with a premutation and a full mutation.

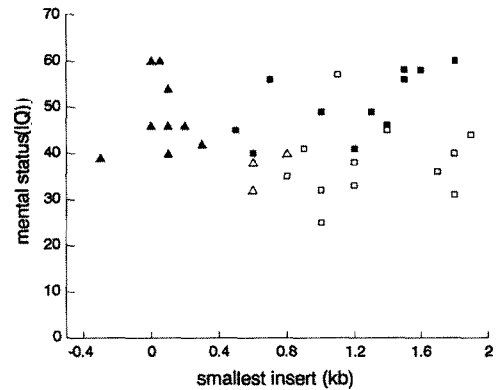


Fig. 4. Smallest detectable insert size in the FMR-1 gene related to the IQ level of 35 patients. ■, □ = Patients with a full mutation only; ▲, △ = patients with a premutation and a full mutation. Filled symbols: age < 14 years; open symbols: age ≥ 14 years.

the mental status of the two groups (patients with a full mutation only and patients with an additional premutation) for their mental status.

We estimated the size of the smallest detectable insert by determining the beginning of the smear on Southern blot in patients with a full mutation only. In patients with a premutation we defined the size of the premutation band as the size of the smallest insert. In figure 4, the smallest detectable insert in the FMR-1 gene is shown in relation to the IQ level of the patient. There is no obvious clustering. Patients with an additional premutation were found with a relatively high IQ as well as with a lower IQ ($n = 12$, mean age = 11.6 ± 5.5 years, mean IQ = 45.3 ± 8.8). The same was seen for patients with a full mutation only ($n = 23$, mean age = 14.3 ± 6.8 , mean IQ = 44.1 ± 10.0). The young, prepubertal, patients (< 14 years, $n = 21$) in both groups have a higher mean IQ than the older patients (≥ 14 years, $n = 14$): mean IQ = 48.4

± 9.1 versus 38.7 ± 6.9 . This age effect on IQ has been reported before [13]. Looking at the two different age groups separately, a correlation between the smallest insert in the FMR-1 gene and IQ could still not be observed. Therefore the IQ level seems not to be related to the smallest insert in the FMR-1 gene in patients. A similar pattern was observed when the mean insert size of the full mutation in the FMR-1 gene is used instead of the smallest insert (data not shown).

In males with a full mutation only, the CpG island proximal to the FMR-1 gene is fully methylated (fig. 2, [9]) and the FMR-1 gene is inactivated [27]. The CpG island proximal to the FMR-1 gene in premutation alleles is unmethylated; incomplete methylation at the CpG island may, therefore, have an effect on the level of mental retardation in these patients. The FMR-1 gene with the unmethylated CpG island still produces mRNA [27] and through this a normal protein may be produced; therefore a difference in mental

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1      CTGCA GAAAT GGGCG TTCTG GCCCT CCGCA GGCAG TGCBA CCTGT CACCG CCCTT CAGCC
61     TTCCC GCCCT CCACC AAGCC CCGCG ACGCC CGGCC CGCGC GTCTG TCTTT CGACC CGGCA
121    CCCC GCGGG TTCCC ACGAG CCGCG ATGCG CCGCG TCCCA GGCCA CTTGA AGAGA GAGGG
181    CGGGG CCGAG GGGCT GAGCC CCGCG GGGGA GGGAA CAGCG TTGAT CACGT GACGT GGTTT
241    CAGTG TTTAC ACCCG CAGCG GGGCG GGGGT TCGGC CTCAG TCAGG CGCTC AGCTC CGTTT
301    CGGTT TCACT TCCGG TGGAG GCGCG CCTCT GAGCG GCGGG CCGGC CGACC GCGAG CCGCG
361    GCGGC GCGGG TGACG GAGGC GCCGC TGCCA GGGGG CGTGC GGCAG CCGGG CCGCG CCGGC
421    GCGGG CCGGG GCGGG GCGGG CCGGG GCGGG GCGGG CTGGG CCTCG AGCGC CCGCA GCCCA
481    CCTCT CGGGG GCGGG CTCCC GCGGC TAGCA GGGCT GAAGA GAAGA TGGAG GAGCT GGTGG
541    TGGAA GTGCG GGGCT CCAAT GCGCG TTTCT ACAAG GTACT TGGCT CTAGG GCAGG CCCCA
601    TCTTC GCCCT TCCTT CCCTC CTTT TCTTC TCGGT GTCGG CGGGA GGCAG GCCCG GGGCC
661    CTCTT CCGGA GCACC GCGCC TGGGT GCCAG GGCAC GCTCG GCGGG ATGTT GTTGG GAGGG
721    AAGGA CTGGA CTTGG GGCCT GTTGG AAGCC CCTCT CCGAC TCCGA GAGGC CTTAG CGCCT
781    ATCGA AATGA GAGAC CAGCG AGGAG AGGGT TCTCT TTCGG CCGCG AGCCC GCGCG GGGTG
841    AGCTG GGGAT GGGCG AGGGC CCGCG GCAGG TACTA GAGCC GGGCG GGAAG GGCCG AAATC
901    GGCBC TAAGT GACGG CGATG GCTTA TTCCC CCTTT CCTAA ACATC ATCTC CCAGC GGGAT
961    CGGGC CCGGT CGTGT GGGTA GTTGT GGAGG AGCGG GGGGC GCTTC AGCCG GCGCG CCTCC
1021   TGCAG

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Fig. 5. DNA sequence of the PstI fragment containing the CGG repeat [12]. The CGG repeat is underlined. The box indicates the deleted basepairs in the FMR-1 gene in the patient.

functioning between both groups might be expected. This difference is not observed.

Several explanations can account for the fact that patients with partial transcription of the FMR-1 gene are not less retarded than the patients with no transcription at all. Firstly, the level of transcription may not reach the necessary threshold to provide normal levels of protein. The protein might be cell-bound so that low levels within deficient cells cannot be compensated by normal levels in protein-producing cells. In addition, the expression is only found in a minority of cells; so the absence of expression in a large number of cells may have a dominant effect, resulting in the fragile X phenotype. Secondly, mRNA expression does not necessarily ensure a normal FMR-1 protein. Thirdly, studies of lymphocytes may not be representative for other tissues. The mRNA expression could still be absent in the appropriate tissue (e.g. brain) or at the stage critical for the development of the fragile X phenotype.

Although the numbers are small our data suggest no relation between partial transcription of the FMR-1 gene and the mental status of the patient. It seems therefore that the presence of the full mutation in the FMR-1 gene is decisive for mental impairment. This feature distinguishes the fragile X syndrome from myotonic dystrophy. In both disorders enlargements of DNA fragments, in the disease locus, are found in affected individuals [28–30]. However, in myotonic dystrophy, the increase in size seems to correspond with increasing severity of the disease within families.

During several years, a follow-up IQ testing was performed, so the IQ development could be observed. In the study group 7 patients (mean age 15.7 ± 2.7 years) had a significant IQ decline (> 16 points) and their mutation pattern was studied. Five patients with a significant IQ decline had only a full mutation. The remaining two patients had a premutation in addition to a full mutation. So

a significant IQ decline is not restricted to patients with a full mutation only, but can occur in patients with an additional premutation as well.

One patient was observed with a deletion in the FMR-1 gene in a part of the cells in addition to a full mutation. Deletions in the FMR-1 gene have not been reported before. The DNA analysis of the patient with a deletion of 250 bp is shown in figure 2 (lane 8); the fragment with the deletion was not methylated. The deletion is located around the CGG repeat (fig. 5). Proximal to the CGG repeat 53 bp are deleted and distal to the repeat 178 bp are deleted. The patient showed expression of the fragile site Xq27.3 in 17% of the blood lymphocytes and had an IQ level of 39 points. On physical examination, he showed the following features: short stature, obesity, short broad hands and feet and hypogenitalism with hyperpigmentation of the genitals. This pattern of features has been described before in two other fragile X boys [31] and has been designated as the 'Prader-Willi-like' subphenotype of the fragile X syndrome. Besides a normal allele, the mother had an allele with a premutation, no deletion was detected.

Several conclusions can be drawn from this study. Firstly, in patients with an addi-

tional premutation the percentage of fragile X expression is not significantly lower compared to patients with a full mutation only. Our data suggest an age-dependent process whereby in adult male patients the number of cells carrying a premutation tends to diminish due to continued mitotic instability in life.

Secondly, the mean insert size of the full mutation in the FMR-1 gene positively correlates with the percentage of fragile X expression. This suggests that the size of CGG repeat in the FMR-1 gene is a casual factor in the generation of the fragile site at Xq27.3 and that expression of the fragile size increases with the number of CGG repeats.

Thirdly, the presence of the full mutation seems decisive for the mental impairment. So males who have a premutation in addition to a full mutation seem as severely mentally retarded as males with the full mutation only.

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